DNA DEPENDENT PROTEIN KINASE CATALYTIC SUBUNIT PHOSPHORYLATION SITES AND ANTIBODIES THERETO

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims priority to U.S. Provisional Patent Application No. 60/375,094, which was filed on April 22, 2002, which is incorporated by reference in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

[002] Applicants assert that the attached paper copy of the Sequence Listing for the utility application, "DNA Dependent Protein Kinase Catalytic Subunit Phosphorylation Sites and Antibodies Thereto," claiming priority to U.S. Provisional Patent Application No. 60/375,094, filed on April 21, 2003, is identical to the Sequence Listing in computer readable form found on the accompanying computer disk, as required by 37 CFR 1.821(c) and is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[003] This invention generally relates to the field of cancer treatment, therapeutics and diagnostics. More specifically, the invention describes antibodies and a method useful for increasing the radiation sensitivity of cancer cells. The invention also provides methods of designing inhibitors of DNA-PKcs that are more specific and result in less harmful side effects.

DESCRIPTION OF THE RELATED ART

[004] In the clinical setting, the two most common treatments for cancer patients are a drug regimen or treatment with high doses of radiation, or a combination of both. Both approaches kill cancerous (and healthy) cells through a common mechanism of inducing DNA damage. DNA double-strand breaks (DSB) are the most common type of DNA damage resulting from either treatment. In human cells, DNA DSBs are repaired mainly by the non-homologous end-joining pathway (NHEJ). The DNA-dependent protein kinase complex (DNA-PK) is a key player in the repair of DNA DSBs by this pathway. If DNA-PK is defective, cells are unable to repair DNA DSBs, and thus become highly sensitive to the

effects of ionizing radiation and of various cancer drugs. Since DNA-PK is a protein kinase, it is able to transfer phosphate groups to target proteins, and thereby regulate their function. DNA-PK is a protein complex consisting of its DNA-binding and regulatory subunit, which is the Ku protein, and the catalytic subunit, called DNA-PKcs. In the presence of DNA DSBs, Ku binds to the ends of the DNA and recruits DNA-PKcs to the site of the DSB. Once bound to Ku and DNA, DNA-PKcs becomes activated and is capable of phosphorylating target proteins.

[005] Although the biochemical properties of DNA-PK have been extensively studies *in vitro*, very little is known about how DNA-PK functions *in vivo* in relation to the repair of DNA DSBs. This lack of progress in studying the physiological functions of DNA-PK is in part due to the unavailability of the right tools or assays to evaluate DNA-PK *in vivo* activity.

[006] Currently, one of the most commonly used methods to study DNA repair proteins is by immunofluorescence with an antibody to the protein of interest. In response to DNA damage, many of the DNA repair proteins form "foci" that can be visualized with antibodies. It is generally believed that these DNA damage-induced foci correspond to sites where the damages DNA is actively being repaired.

[007] It is currently not possible to detect DNA-PK foci with the antibodies available because DNA-PK is quite abundant in the nucleus, thus when one performs immunofluorescence with any of the available antibodies, the entire nucleus will produce a signal, making it impossible to see any discernable foci. Therefore, it is of interest to develop an antibody that can overcome the problem associated with a very high background signal and can recognize the phosphorylated form of DNA-PKcs when bound to site of DNA DSBs.

[008] DNA-PK is a serine/threonine protein kinase that *in vitro* is activated by DNA ends and has long been established to play an important role in the repair of DNA double-strand breaks (DSB) by the NHEJ pathway (Smith and Jackson, *Genes Dev.* 1999 Apr 15;13(8):916-34). DNA-PK is capable of autophosphorylating the two Ku subunits, Ku70 and Ku80 according to Chan et al., *Biochemistry* 1999 Feb 9;38(6):1819-28. Autophosphorylation of DNA-PKcs causes it to dissociate from Ku, resulting in the loss of kinase activity (Chan and Lees-Miller, J Biol Chem. 1996 Apr 12;271(15):8936-41). In addition, the inventors have shown that the kinase activity of DNA-PKcs is absolutely required for its function in the NHEJ pathway since a DNA-PKcs-deficient CHO cell line expressing a kinase dead form of DNA-PKcs was incapable of repair (Kurimasa et al., *The Journal of Immunology*, 2000, 165: 3883-3889). Therefore, the kinase activity of DNA-PK is

absolutely required for the repair of DNA DSBs; however, the molecular mechanism of this requirement for kinase activity remains to be elucidated. DNA-PK is also capable of autophosphorylation, that is, it transfers phosphate groups onto itself, and that autophosphorylation may be an important mechanism for regulating its kinase activity (Kurimasa et al., *Molecular and Cellular Biology*, May 1999, p. 3877-3884, Vol. 19, No. 5).

[009] DNA-PKcs is an extremely large protein consisting of 4129 amino acids, and therefore identifying the site of autophosphorylation is comparable to finding a very small needle in a large haystack. Cloning of the DNA-PKcs cDNA is difficult, since the cDNA exceeds 13 kb. In the past, using classical biochemical techniques, several labs have attempted but failed to identify the autophosphorylation sites. For example, *in vivo* radiolabelling with ^{32}P and 2-dimensional phosphopeptide mapping failed to identify any autophosphorylation sites.

[010] One goal of radiation biology is to find ways to increase the radiation sensitivity of cancer cells. If this could be achieved, it would then be possible to treat cancer patients with lower doses of radiation and thereby dramatically decrease the side effects and complications associated with radiation treatment.

[011] If the site of phosphorylation in DNA-PK could be specifically blocked in cancer cells, for example with a DNA-PKcs inhibitor, then this should inhibit DNA-PKcs-mediated repair of DNA DSBs and thereby increase the radiation sensitivity of the treated cancer cells. Another possible means of increasing radiation sensitivity is the development of therapeutic antibodies that can specifically recognize and bind to the phosphorylated protein.

BRIEF SUMMARY OF THE INVENTION

[012] The present invention is directed to the identification and use of two major DNA-PKcs autophosphorylation sites, Threonine (T) 2609 and Serine (S) 2056, including antibodies specific for phosphorylated T2609 and S2056. It is demonstrated that phosphorylation of these sites, carried out *in vivo* by the DNA-PKcs itself (i.e. autophosphorylation), is required for DNA-PK activity and, furthermore, that such activity repairs double strand DNA breaks (DSBs) and improves cell survival to ionizing radiation (IR). For example, it is demonstrated a point mutation at position 2056 from serine to alanine and position 2609 from threonine to alanine results in cells that are radiosensitive.

[013] The present invention further provides phosphospecific antibodies that recognize these specific sites of phosphorylation in DNA-PKcs. The antibodies do not bind to the unphosphorylated DNA-PKcs protein or peptide. This provides diagnostic tools based

on the ability to identify the phosphorylation status of the DNA-PKcs autophosphorylation sites. One can monitor the effectiveness of treatments which target the DNA repair pathway of cancer cells, such as radiation treatment and inhibitor drugs. Also, the ability to intervene in autophosphorylation of T2609 or S2056, either through application of a drug or an antibody, would increase the radiation-induced killing of cancer cells.

[014] In one embodiment there is provided an antibody which specifically binds to an epitope defined by at least a ten amino acid sequence from DNA-PKcs and comprising a phosphorylated threonine at position T2609 in human DNA-PKcs, which antibody does not bind when T2609 is not phosphorylated. The antibody may be an affinity purified polyclonal antibody or a monoclonal antibody. The monoclonal may be a conventional hybridoma produced mouse monoclonal, or may be a human monoclonal produced by known techniques. In one embodiment, the human monoclonal is produced using a mouse with a human immune system as an immune cell donor in a hybridoma process. One specific embodiment is the pT2609 monoclonal antibody, pT2609mAb.

[015] The invention further comprises an antibody which specifically binds to an epitope defined by at least a ten amino acid sequence from DNA-PKcs and comprising a phosphorylated serine at position S2056 in human DNA-PKcs, which antibody does not bind when S2056 is not phosphorylated. Again, the antibody may be an affinity purified polyclonal antibody or a monoclonal.antibody. The monoclonal may be a conventional hybridoma produced mouse monoclonal, or may be a human monoclonal produced by known techniques. In one embodiment, the human monoclonal is produced by using a mouse with a human immune system as an immune cell donor in a hybridoma process. One specific embodiment is the pS2056 monoclonal antibody, pS2056mAb.

[016] In any case, the binding epitope is contained both on full length DNA-PKcs and subsequences thereof, said subsequences having at least about 10 amino acids.

[017] The present invention further comprises methods for determining the ability of a test compound to block phosphorylation of human DNA-PKcs. One method comprising the following steps: (a) providing a sample containing a DNA-PKcs peptide fragment capable of being phosphorylated; (b) adding the test compound to the sample; (c) inducing phosphorylation of the DNA-PKcs protein in the sample; and (d) measuring the resulting phosphorylation of DNA-PKcs at T2609 or S2056 in the presence of the test compound. This is preferably done in comparison to a sample containing a DNA-PKcs peptide fragment which is phosphorylated in the absence of the test compound.

[018] The method may also involve providing a sample containing an artificial peptide containing the T2609 and/or S2056 site. Recombinant DNA-PK or DNA-PKcs is added to the mixture and will phosphorylate the artificial peptide. The artificial peptide may be on the order of about 1000 amino acids long or as short as 20 amino acids long.

[019] The measuring step may be carried out by measuring the binding of an antibody which specifically binds to an epitope comprising either or both of (a) a phosphorylated serine at position S2056 in human DNA-PKcs or (b) a phosphorylated threonin at position T2609 in human DNA-PKcs

[020] Known kinase inhibitors provide suitable starting points for assaying test compounds that are capable of blocking or inhibiting phosphorylation of DNA-PKcs. In this assay, test compounds are any organic molecules that are capable of blocking or inhibiting phosphorylation of DNA-PKcs. Non-limiting examples include wortmannin, substituted or unsubstituted imidazoles, pyrazoles, benzofluoranthenes, thiazoles, isoquinolinones, dihydrolisoquinolinones, phthalazinones and related compounds and derivatives thereof. For example, since wortmannin has been shown herein to inhibit the phosphorylation of T2609 and S2056, derivatives and analogs of wortmannin provide sources of test compounds to be tested in the present assay. Functional groups could be introduced into the wortmannin structure adjacent to the heterocyclic oxygen adjacent to C21 or the double bond between C4 and C21.

[021] To identify drug inhibitors of DNA-PK, one first initially screens available chemical libraries for test compounds that could inhibit DNA-PK kinase activity *in vitro* or organic molecules that are capable of blocking or inhibiting phosphorylation of DNA-PKcs. The compounds in these chemical libraries can be added to *in vitro* DNA-PK kinase assays to identify the ones that could inhibit DNA-PK activity. Because phosphorylation of T2609 and S2056 is via an autophosphorylation mechanism, it can be speculated that any drug compounds that inhibit the kinase activity will inhibit the autophosphorylation of these two sites. Once these compounds have been identified, cellular studies can then be carried out to evaluate their efficacy.

[022] Further aspects of the present invention involve phosphopeptides that have been prepared for use in injection into animals in the course of antibody preparation (haptens) or for use as artificial phosphorylation substrates. These peptides will have less than 30 amino acids and comprise SEQ ID NO: 1 or SEQ ID NO: 2, or sequences having at least 90% homology thereto having the requisite serine or threonine residues, preferably in an SQ or TQ

sequence. These phosphopeptides may also have an amino acid other than the wild type T2609 or S2056, to serve as negative controls.

[023] The above described isolated peptides have further utility when T2609 and/or S2056 is replaced by an amino acid which is not phosphorylated, such as: Valine, Alanine, Glycine, or Leucine. These embodiments serve as negative controls and will inhibit phosphorylation.

[024] The above described peptides may be encoded by an isolated polynucleotide cloned and inserted into a suitable host vector.

[025] The present invention further comprises a method of measuring radiosensitivity of cells in a subject undergoing radiation treatment. This method comprises the steps of (a) providing a cell sample from said subject and containing DNA-PKcs, for example a blood sample or a tissue sample from the irradiated area (e.g. a nuclear extract may be prepared from this sample); (b) adding to said sample a labeled antibody which binds to phosphorylated residue T2609 or phosphorylated residue S2056 but not the unphosphorylated residues; (c) removing unbound antibody from the sample, such as by washing, as is known in the art; and (d) measuring the degree of phosphorylation of the DNA-PKcs by determining the amount of antibody bound to the DNA-PK in the sample. The degree of antibody binding to DNA-PKcs in the cell sample correlates to the degree of phosphorylation, a higher degree of phosphorylation indicating less radiation sensitivity

[026] Since most treatment for cancer entails inducing DNA damage, a pT2609 or pS2056 antibody can be a very useful diagnostic tool for determining the efficacy of the treatment. For example, the antibody can be used to confirm that the cancer treatment is indeed causing DNA damage in the cancer cells; conversely, the antibody can be used to determine the effects of the treatment on healthy cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[027] **Fig. 1** is a mass spectra of sequencing of the *in vitro* phosphorylated DNA-PKcs peptide that was first identified by MALDI-TOF mass spectrometry. The plot shows a plot of relative intensity *vs* the mass-to-charge ratio (m/z) of the phosphorylated peptide having the shown sequence from 2599 to 2619. Assignment of all the mass spectra peaks unequivocally identify T2609 as the site of phosphorylation.

[028] **Fig. 2** is the mass spectra of phosphatase-treated (bottom trace) and the mock treated (top trace) DNA-PKcs peptides from irradiated HeLa cells which were purified by immunoprecipitation and digested with Asp-N protease, then analyzed by MALDI-TOF mass

spectrometry. The loss of the peak with the m/z of 3511 with phosphatase treatment, and the presence of the peak corresponding to the unphosphorylated peptide (m/z of 3433) allowed the positive assignment of a phosphorylation site to the sequence of DNA-PKcs between amino acids 2044-2072.

[029] **Fig. 3A** is a Western blot showing DNA-PKcs protein expression levels of vector along (V3-JM), full-length wild-type DNA-PKcs (V3-F18) and T2609A mutant of DNA-PKcs in V3 cell line (top). Hamster Werner proteins were analyzed to show equal sample loading (bottom). **Fig. 3B** is a graph showing that the T2609A, S2056A and the T2609A/S2056A double mutation increase cellular radiation sensitivity. **Fig. 3C** is a graph showing that the T2609A and S2056A mutations compromise DSB repair in cells as determined by the fraction of activity released (FAR) assay.

[030] **Fig. 4A** is two Western blots showing that the pT2609pAb is specific for the phosphorylated T2609 site in wild type DNA-PKcs. **Fig. 4B** is two Western blots showing that the pT2609 polyclonal antibody (pT2609pAb) does not recognize unphosphorylated DNA-PKcs at the molar ratios given as compared to 25-4 DNA-PKcs monoclonal antibody (25-4 mAb). **Fig. 4C** is two Western blots showing that the pS2056 polyclonal antibody (pS2056pAb) does not recognize unphosphorylated S2056 in DNA-PKcs as compared to 25-4 DNA-PKcs monoclonal antibody (25-4 mAb).

[031] **Fig. 5A** is a Western blot of Hela cell nuclear extracts, probed with pT2609 antibody (upper panel) and 25-4 monoclonal antibody to DNA-PKcs (bottom panel), after either mock-treatment or irradiation with 10 Gy and recovery for various times. **Fig. 5B** is a Western blot of Hela cell nuclear extracts, probed with pT2609pAb (upper panel) and 25-4 monoclonal antibody to DNA-PKcs (bottom panel), after irradiation at the indicated dose and recovery for 30 minutes. **Fig. 5C** is a Western blot showing that pT2609 polyclonal antibody can be used to immunprecipitate phosphorylated DNA-PKcs from unirradiated HeLa nuclear extract (lane 1) but not unphosphorylated DNA-PKcs from extracts made from HeLa cells irradiated with 25 Gy and harvested after 30 min recovery period. The 25-4 monoclonal antibody does not discriminate between phosphorylated or unphosphorylated DNA-PKcs. **Fig. 5D** shows by Western blot that phosphorylation of T2609 in response to DNA damage can be inhibited with wortmannin treatment (left panel) and is inducible in A-T cells (right panel).

[032] **Fig 6A** is a Western blot showing that phosphorylation of S2056 is IR-inducible. **Fig. 6B** is a time course Western blot of IR-inducible phosphorylation of Ser 2056.

DETAILED DESCRIPTION OF THE PREFFERRED EMBODIMENT

A. Definitions

[033] "Radiosensitization" herein refers to a means of increasing the sensitivity of human cells to the effects of ionizing radiation and cancer drugs that induce DNA double-strand breaks (DSBs). By increasing the radiosensitivity of cancer cells, patients can be treated with lower doses of radiation or chemotherapeutic regimen and thereby decrease the harmful side effects of the treatment.

[034] "Gy" herein refers to describe the unit (SI unit) of absorbed dose of radiation (Gy), wherein 1 Gy = 1 J kg^{-1} = 100 rad.

[035] "Epitope" has its conventional meaning, i.e. a single antigenic determinant. Functionally it is the portion of an antigen (e.g. a PKcs peptide) which combines with the antibody paratope. Structurally, it is the specific amino acid residues or portions thereof to which an anti-peptide antibody binds.

[036] "DNA-PKcs" herein refers to DNA-dependent protein kinase catalytic subunit (EC 2.7.1.37), preferably human DNA-PKcs. As used herein, the numbering is based on GenBank Accession Number P78527, as set out in SEQ ID NO: 3. Specifically, T2609 is threonine 2609 in Genbank Accession Number P78527. S2056 is serine 2056 in GenBank Accession Number P78527. DNA-PK refers to the entire enzyme. DNA-PKcs is encoded by the nucleotide sequence as set out in SEQ ID NO: 15, having GenBank Accession Number U47077.

[037] "Monoclonal antibody" has its conventional meaning, and is explained more fully in U.S. patent 4,619,895, hereby incorporated by reference for purposes of describing preparation and characterization of mouse monoclonal antibodies, U.S. patent 4,744,982 hereby incorporated by reference for purposes of describing human/human monoclonal antibody preparation and characterization, U.S. patent 5,874,540, hereby incorporated by reference for purposes of describing the preparation and characterization of CDR-grafted humanized antibodies, and U.S. patent 6,075,181, hereby incorporated by reference for purposes of describing the preparation and characterization of human antibodies derived from immunized xenomice.

[038] The abbreviation "mAb" herein refers to monoclonal antibodies and the abbreviation "pAb" herein refers to polyclonal antibodies.

[039] "Humanize," when applied to antibodies, herein refers to methods of generating human monoclonal antibodies, as exemplified by van de Winkel, in U.S. Pat. No. 6,111,166, hereby incorporated by reference for purposes of describing such methods.

[040] "Isolated," when applied to a polynucleotide, herein refers to that the polynucleotide has been removed from its natural genetic milieu and is thus free of the extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA, synthetic DNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, *Nature* 316:774-78, 1985).

[041] "Isolated," when applied to a polypeptide or protein, herein refers to a polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

[042] "Polynucleotide" herein refers to a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 51 to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized in vitro, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

[043] "Polypeptide" herein refers to a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

[044] "Homologous" herein refers to the sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules or two RNA molecules, or between two peptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between-two sequences is a direct function of the number of matching or homologous positions, e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3' ATTGCC 5' and 3' TATGCG 5' share 50% homology. Any of a variety of known algorithms may be used to calculate the percent homology between two nucleic acids or two proteins of interest and these are well-known in the art.

[045] "Substantial homology" or "substantial identity", when referring to polypeptides, herein refers to that the polypeptide or protein in question exhibits at least about 30% identity using BLASTP (Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410) with an entire naturally-occurring protein or a portion thereof, usually at least about 70% identity over the common lengths, more usually at least about 80% identity, preferably at least about 90% identity, and more preferably at least about 90% identity or 90% positive, whichever is less. For purposes of calculating homology between two polypeptides, the standard BLASTP 2.2.5 defaults are used, namely "Expect 10," "Word size 3," "BLOSUM62 Matrix" and "Gap Costs Existence10, Extension 1."

[046] In this specification, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

B. Role of DNA PK-cs in Double Strand Break Repair

[047] Repair of DNA double strand breaks (DSBs) in mammalian cells is mainly mediated by the non-homologous end-joining pathway (NHEJ). The DNA-dependent protein kinase (DNA-PK) complex play critical roles in the NHEJ pathway since mammalian

cell lines that lack components of the complex show severe radiation sensitivity and DNA repair defects. DNA-PK is a serine/threonine protein kinase that is activated by the DNA ends *in vitro* and is composed of the DNA-binding and regulatory subunit, Ku, and the catalytic subunit, DNA-PKcs. DNA-PK kinase activity is required for the repair of DNA DSBs *in vivo* (Kurimasa et al., *Mol Cell Biol.* 1999 May; 19(5):3877-84). Previously, it was demonstrated that DNA-PK activity is negatively regulated by an autophosphorylation mechanism *in vitro*. (Chan et al., *Biochem Cell Biol* 74: 67-73, 1996).

[048] In the preferred embodiment, the DNA-PKcs being investigated has an amino acid sequence comprising SEQ ID NO: 3 and wild-type cDNA sequence comprising SEQ ID NO: 15.

[049] Herein is reported the method and identification of residues of DNA-PKcs which can act as major *in vitro* and *in vivo* autophosphorylation sites.

C. Autophosphorylation Sites in DNA-PKcs

[050] Determination of autophosphorylation sites in DNA-PKcs is preferably done by mass spectrometry. For example, purified DNA-PKcs and Ku can be autophosphorylated by adding a low concentration of ATP to allow phosphorylation of the most preferential site and then the autophosphorylated DNA-PKcs was analyzed by mass spectrometry. Alternatively, DNA-PKcs can be immunoprecipitated from nuclear extracts prepared from irradiated HeLa cells and then analyzed by mass spectrometry.

[051] As described below, two major *in vitro* and *in vivo* autophosphorylation sites of the residues of T2609 and S2056 were identified by mass spectrometry. Purified DNA-PKcs and Ku were autophosphorylated with low concentration of ATP (50uM) to allow phosphorylation of the most preferential site. Referring now to Fig. 1, the *in vitro* autophosphorylated DNA-PKcs was analyzed by mass spectrometry and T2609 was unambiguously identified as a major site of autophosphorylation. T2609 lies in a region of DNA-PKcs that is not conserved between the various members of the phosphatidylinositol 3-kinase (PI-3) family members, to which DNA-PKcs is a member of. However, T2609 was absolutely conserved in all known DNA-PKcs homologues found in GenBank (i.e. mouse, dog, horse, chicken and xenopus) when the sequences are compared. This suggests the significance of the phosphorylation of this residue and that phosphorylation of DNA-PKcs at this residue maybe be conserved throughout evolution.

[052] Referring now to Fig. 2, a second major *in vitro* and *in vivo* autophosphorylation site of DNA-PKcs, S2056, was identified by immunoprecipitating DNA-PKcs from nuclear extracts prepared from irradiated HeLa cells and analyzed by mass

spectrometry. As described in detail below, mass spectrometry identified the following phosphopeptide sequence, DFSTGVQSYSYSSQDPRPATGRFRRREQR (SEQ ID NO: 5), which corresponds to amino acids 2044 to 2072 of DNA-PKcs (S2056 is underlined). Upon careful analysis of the sequence, S2056 proved to be the site of phosphorylation. This is consistent with prior suggestions that DNA-PK preferentially phosphorylates "SQ" and "TQ" sequences and S2056 followed this "SQ" consensus sequence. Similar to T2609, the sequence alignment with other vertebrate DNA-PKcs homologues in GenBank shows that amino acids 2044 to 2056 in DNA-PKcs are highly conserved throughout evolution in vertebrates.

D. Role of DNA-PKcs Autophosphorylation Sites in Radiation Sensitivity

[053] To investigate the biological significance of the autophosphorylation of these DNA-PKcs sites in relation to DNA repair, wild-type or mutant DNA-PKcs having the site of autophosphorylation mutated were transfected into the DNA-PKcs-defective V3 cell line (Kurimasa et al., *J Immunol.* 2000 Oct 1;165(7):3883-9). The resulting V3 cell lines were isolated and evaluated for DNA-PKcs protein expression levels, radiation sensitivity, and DNA repair defects. Cells expressing the mutant DNA-PKcs protein exhibit a more severe radiation sensitivity phenotype as compared with wild-type DNA-PKcs protein yet not as severe as the V3 cell line that totally lacks DNA-PKcs. The V3-mutant DNA-PKcs cells exhibits a radiation sensitivity phenotype of about a 10 fold increase in cell death at 5 Gy when compared with V3-wild type cells, demonstrating a dramatic difference in radiation sensitivity in mammalian cells.

E. Generation of anti-pT2609 and anti-pS2056 phosphospecific antibodies

[054] To study the *in vivo* phosphorylation status of DNA-PKcs at the autophosphorylation sites, a phosphsphospecific antibody that recognizes the phosphorylated residue of DNA-PKcs is generated, then affinity purified to insure specificity.

[055] DNA-PKcs phosphospecific antibodies can be made by a number of methods known in the art. These phosphospecific antibodies include antibodies which recognize phosphorylated T2609, herein referred to as pT2609 antibodies and phosphorylated S2056, herein referred to as pS2056 antibodies. A preferred method is by generating phosphopeptides. These phosphopeptides can be synthesized or produced by first amplifying and cloning cDNA fragments of SEQ ID NO: 15, the cDNA sequence of human DNA-PKcs (GenBank Accession No. U47077), and then expressing peptide fragments of DNA-PKcs from the cloned cDNAs. These phosphopeptide fragments include the site of

autophosphorylation and the adjacent DNA-PKcs amino acid sequence on either side of the position being autophosphorylated. It is preferred that at least 6, preferably no more than 10 amino acids of the wild-type DNA-PKcs protein sequence are used on either side of the phosphorylation site to generate very specific antibodies. Two such preferred phosphopeptides are SEQ ID NO: 1 and 2 and shown below.

SEQ ID NO: 1 N'--TPMFVET*QASQGTC--C' (* indicating phospho group at T2609)

SEQ ID NO: 2 N'--QSYSYSS*QDPRPAC--C' (* indicating phospho group at S2056)

[056] Since synthesized phosphopeptides are not always immunogenic on their own, the peptides were conjugated to a carrier protein before use. Appropriate carrier proteins include, but are not limited to, Keyhole limpet hemacyanin (KLH), bovine serum albumin (BSA) and ovalbumin (OVA). The conjugated phosphopeptides should then be mixed with adjuvant and injected into a mammal, preferably a rabbit through intradermal injection, to elicit an immunogenic response. Samples of serum can be collected and tested by ELISA assay to determine the titer of the antibodies and then harvested.

[057] Polyclonal pT2609 and pS2056 antibodies can be purified by passing the harvested antibodies through an affinity column. However, monoclonal antibodies are preferred over polyclonal antibodies and can be generated according to standard methods known in the art of creating an immortal cell line which expresses the antibody.

[058] Nonhuman antibodies are highly immunogenic in human thus limiting their therapeutic potential. In order to reduce their immunogenicity, nonhuman antibodies need to be humanized for therapeutic application. Through the years, many researchers have developed different strategies to humanize the nonhuman antibodies. One such example is using "HuMAb-Mouse" technology available from MEDAREX, Inc. (Princeton, NJ). "HuMAb-Mouse" is a strain of transgenic mice that harbors the entire human immunoglobin (Ig) loci and thus can be used to produce fully human monoclonal pT2609 and pS2056 antibodies.

[059] Immunoblotting using the phosphospecific antibodies of the invention with unphosphorylated DNA-PKcs should not produce a detectable signal at preferably 0.5-10 fold molar excess (relative to the phosphorylated DNA-PKcs), more preferably at 50 fold molar excess and most preferably no signal is detected at even 100 fold molar excess.

F. Designing and Making DNA-PKcs Inhibitor Drugs

[060] The phosphorylation of DNA-PKcs at Threonine 2609 and Serine 2056 is required for the repair of DNA double strand breaks. By inhibiting the phosphorylation of

these two sites with small molecules, it may be possible to increase the radiation-induced killing of cancer cells.

[061] To identify drug inhibitors of DNA-PK, one first initially screens available chemical libraries for test compounds that could inhibit DNA-PK kinase activity *in vitro* of organic molecules that are capable of blocking or inhibiting phosphorylation of DNA-PKcs. Analysis of known kinase inhibitors provides suitable starting points and non-limiting examples include wortmannin, substituted or unsubstituted imidazoles, pyrazoles, benzofluoranthenes, thiazoles, isoquinolinones, dihydrolisoquinolinones, phthalazinones and related compounds and derivatives thereof. The compounds in these chemical libraries can be added to *in vitro* DNA-PK kinase assays to identify the ones that could inhibit DNA-PK activity. Because phosphorylation of T2609 and S2056 is via an autophosphorylation mechanism, it can be speculated that any drug compounds that inhibit the kinase activity will inhibit the autophosphorylation of these two sites. Once these compounds have been identified, cellular studies can then be carried out to evaluate their efficacy.

[062] Amino acid peptide fragments of DNA-PKcs around the T2609 and S2056 sites were expressed in *E.coli* despite the difficulty encountered in cloning the corresponding cDNA sequence. In a preferred embodiment, the DNA-PKcs cDNA clones containing SEQ ID NO: 18 and SEQ ID NO: 22, which express peptide fragments corresponding to the amino acid sequence of DNA-PKcs from residues 1879-2182 and from 2500-2700 respectively, are made. In another preferred embodiment, an 822 amino acid fragment (residues 1879-2700) can be expressed from a cDNA clone containing SEQ ID NO: 20 because this fragment once expressed encompasses both phosphorylation sites.

[063] These fragments which encompass one ot or both phosphorylation sites can be made by amplifying the appropriate cDNA sequence from a full-length DNA-PKcs cDNA (SEQ ID NO: 15, GenBank Accession No. U47077) by PCR, then cloning and expressing the cDNA sequence to generate the peptide fragment. Primers can be designed and made from SEQ ID NO: 15. It is preferred that the peptide fragment containing the phosphorylation site, be of a length of at least 10, preferably 100, and more preferably about 1000 amino acids of the DNA-PKcs protein sequence.

[064] In one embodiment, these fragments can be used to test how effectively potential drugs inhibit the phosphorylation and activation of DNA-PKcs. Recombinant fragments containing these two phosphorylation sites can be used as molecular targets for small molecular screening. Specifically, small molecules which can bind to these fragments with high affinity will be identified. The inhibition capability of these small molecules can

be verified by their ability to block T2609 and S2056 phosphorylation. The radiation sensitization ability of these small molecules can then verified in human cells upon radiation damage.

[065] In addition, fragments that may also be useful can be expressed from the following clones. These clones were made to express the following residues of DNA-PKcs from the indicated corresponding cloned cDNA sequence: 1879-2182 cDNA (SEQ ID NO: 18), 1879-2267 cDNA (SEQ ID NO: 19), 2261-2700 cDNA (SEQ ID NO: 21), 2275-2702 cDNA (SEQ ID NO: 23), 2429-2702 cDNA (SEQ ID NO: 24), 2561-2700 cDNA (SEQ ID NO: 25), and 2600-2702 cDNA (SEQ ID NO: 26).

G. Peptide Inhibitor Drugs

[066] One embodiment is to use the antibodies of the invention for use as an inhibitor of the phosphorylation and thereby block DNA repair which results in radiosensitization of cancer cells. Because of the specificity of the pT2609 and pS2056 antibodies of the invention, only the sites of phosphorylation are inhibited. Furthermore, phosphorylation of T2609 and S2056 occurs only in cells that have been irradiated and suffer DNA damage. Therefore, the use of the antibodies, or peptide fragments thereof, as DNA repair inhibitors will not affect other proteins or even other parts and functions of the DNA-PKcs protein. This specificity will result in not only radiosensitizing cancer cells, but also this antibody will reduce the other harmful side effects of inhibiting all DNA-PKcs function.

[067] If phosphorylation of T2609 is required for the recruitment of other proteins needed at the site of DNA DSBs, then another means of disrupting this step is to overexpress a small polypeptide spanning the region that surrounds T2609 or S2056 with an Aspartic acid mutation to simulate the phosphorylated state and create a "dominant negative" effect. Therefore, in another embodiment, a polypeptide, such as the peptides generated in Example 2, made with an Aspartic acid or other similarly negatively charged amino acid substituted at residue 2609 or 2056 to mimic the phosphorylated state of T2609, can be overexpressed or administered to compete with endogenous phosphorylated DNA-PKcs. This would "squelch" DNA-PKcs function and therefore lead to an increase in radiation sensitivity.

H. Diagnostic Tools for Detecting Efficacy of Therapeutic Treatments

[068] Companies are developing specific inhibitors for DNA-PKcs or Ku for the purpose of sensitizing cancer cells for radiation therapy. Currently, there is no efficient way to estimate the amount of the inhibitors to be used for sensitization. Antibodies against T2609 or S2506 can be used as a diagnostic tool to effectively monitor blood samples in the test tube to estimate the dose to be used to effectively block the autophosphorylation of

DNA-PKcs. The present pT2609 or pS2056 antibodies can provide a diagnostic tool for determining the efficacy of treatment using DNA-PKcs or Ku inhibitors. The antibodies of the invention can be used to confirm whether the cancer treatment the patient is undergoing is indeed causing DNA damage in the cancer cells; conversely, the antibody can be used to determine the effects of the treatment on healthy cells. Furthermore, diagnostic tests to test the efficacy of inhibitors during drug development can be made based on observations of phosphorylation of T2609, such as in Example 6 or 8.

[069] In one embodiment, the antibodies of the invention can be used to determine the correct radiation dosage for each patient. The normal patient dosage is 2Gy/day up to 50 Gy/day. Since every cancer patient responds to radiation therapy differently, the pT2609 and pS2056 antibodies can be a very useful tool to monitor the effectiveness of the cancer treatment. In a specific embodiment, for example, a small blood sample is drawn from a cancer patient and a quick radiation pulse is applied to the sample to induce DNA damage, then contacting a small volume of the radiated blood with the antibodies of the invention. To increase the signal, the antibodies can be conjugated to another antibody or other means of detection used. Unbound antibodies are washed from the sample and antibodies bound to the patient's DNA-PKcs are measured. A large signal as compared to a control will indicate to an oncologist that any inhibitors that target DNA-PKcs are not working and not inhibiting DNA repair. That is, there is a high degree of DNA-PK autophosphorylation. A low or no signal would indicate that the inhibitors are working which has resulted in the radiosensitization of cancer cells. Alternatively, the blood sample is not irradiated but taken from a patient following radiation treatment to monitor the radiation therapy. In addition, the cells may be taken from a biopsy of the patient's tumor or cancer cells.

[070] In another embodiment, the peptide fragments of SEQ ID NOS: 4-14, would permit the screening of small molecular inhibitors to block phosphorylation at these sites. Small molecular inhibitors which would block the phosphorylation at T2609 or S2506 would be more effective radiosensitizers and have less side effects for radiotherapy. The reason is that the phosphorylation of these two sites only occurs after radiation or DNA damage in response to DSBs and only activated in DNA double-strand break repair. Therefore, inhibitors which are specific for these two sites should not have any effect in cells which have not been irradiated.

EXAMPLE 1

Determining T2609 and S2056 Sites of Autophosphorylation in DNA-PKcs by Mass Spectrometry

[071] First, purified human DNA-PKcs and Ku were autophosphorylated as previously described (Chan and Lees-Miller, *J Biol Chem* **271**: 8936-8941, 1996), and hereby incorporated by reference, with the following change: 50 μM ATP was used instead of 250 μM to allow phosphorylation of the most preferential site. Purified DNA-PKcs and Ku proteins were preincubated at 30 °C. Reactions contained 25 mM Hepes, pH 7.5, 75 mM KCl, 10 mM MgCl, 1 mM dithiothreitol, 0.2 mM EGTA, 0.1 mM EDTA plus 10 μg/ml sonicated calf thymus DNA, and 0.25 mM ATP containing stabilized [-P]ATP (Sigma Chemicals, St. Louis, MO) (specific activity, 500-1000 dpm/pmol) and were started by the addition of purified DNA-PK proteins (usually 0.05-0.1 μg as indicated). Reactions were at 30 °C for 5-10 min and DNA-PK activity was calculated as nmol of phosphate incorporated into the peptide substrate per minute per milligram of protein. Unlabeled ATP or the nonhydrolyzable ATP analogue AMP-PNP (Sigma Chemicals, Stl. Louis, MO) were present where indicated at 50 μM. After 0-10 min, aliquots were removed and analyzed by SDS-PAGE. The band corresponding to phosphorylated DNA-PKcs was excised and digested with trypsin.

[072] The tryptic DNA-PKcs fragments were analyzed by mass spectrometry as previously described by Zhang et al., *Anal Chem* **70**: 2050-2059, 1998. This procedure facilitates the identification of precise phosphorylation sites in proteins separated by polyacrylamide gel electrophoresis by a combination of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF) and on-line capillary liquid chromatography electrospray tandem ion trap mass spectrometry (LC/ESI/MS/MS).

[073] To identify the DNA-PKcs *in vitro* T2609 phosphorylation site, purified DNA-PK was *in vitro* autophosphorylated under the conditions described above and analyzed by SDS PAGE. To identify the *in vivo* S2056 DNA-PKcs phosphorylation site, 10 L of HeLa S3 cells were irradiated with 25 Gy of ionizing radiation. Nuclear extracts made from the irradiated cells and immunoprecipitated, according to the method described in Example 3, with the 25-4 monoclonal antibody to DNA-PKcs (NeoMarkers, Lab Vision, Fremont, CA) and analyzed by SDS PAGE. All chemicals in this analysis were obtained from Fisher Scientific (Pittsburgh, PA).

[074] The coomassie blue-stained DNA-PKcs bands were excised from the gel and destained with 50 mM NH_4CO_3 in 50% methanol. Once destained, the gel slices were fixed overnight with 10% acetic acid and 50% methanol. The gel slices was then swelled with water for 2 hr and grounded to a fine powder in 10 μL of 50 mM NH_4HCO_3. Trypsin (Roche Diagnostics, Alameda, CA) was added and the samples were incubated at 37°C for 90 min.

The digested peptides were extracted from the gel with acetonitrile and concentrated by centrifugation with SpeedVac. A portion of the dried peptides was redissolved in 50 mM NH_4CO_3 for digestion with Asp-N protease (Roche Diagnostics, Alameda, CA) at 37°C for 90 min and dried. The dried tryptic and tryptic-Asp N peptides were dissolved in 10μL of 50% acetonitrile for further processing. A portion of the sample was treated with calf intestine phosphatase (CIP) (New England Biolabs, Beverly, MA) in 50 mM NH_4CO_3 at 37°C for 90 min and dried by centrifugation in a SpeedVac. The dried peptides were redissolved in 2 μL of 50% acetonitrile for MALDI-TOF mass spectrometry.

[075] The CIP-treated and untreated peptides were analyzed in a Voyager DE MALDI-TOF system from Perspective Biosystems. Most of the peaks in the spectrum of the tryptic digest could be easily assigned to unique peptides predicted from the protein sequence and to peptides formed by autolysis of trypsin. Peaks that could not be accounted for in this way were candidates for modified peptides, and those peptides having observed masses that were 80 Da (or multiples of 80 Da) higher than that calculated for a predicted tryptic peptide were tentatively assigned as phosphopeptides. This assignment was confirmed by the absence of these peaks from the MALDI/TOF spectrum of the same peptide mixture after treatment with CIP and the appearance of new peaks that are 80 Da (or multiples of 80 Da) lower in mass. Once the phosphorylated peptide was identified, it was then analyzed on an electrospray ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA) coupled on-line with a capillary HPLC (Magic 2002, Michrom BioResources, Auburn, CA) to identify the phosphorylation sites in the phosphorylated peptides.

[076] Referring now to Fig. 1, there is shown the mass spectra of one of the tryptic fragments. Fig. 1 is a mass spectra of sequencing of the *in vitro* phosphorylated DNA-PKcs peptide that was first identified by MALDI-TOF mass spectrometry. The plot shows a plot of relative intensity *vs* the mass-to-charge ratio (m/z) of the phosphorylated peptide having the sequence (shown in the Figure) from 2599 to 2619. Assignment of all the mass spectra peaks unequivocally identify T2609 as the site of phosphorylation. Thus, T2609 was unambiguously identified as a major site of autophosphorylation (Fig. 1).

[077] Referring now to Fig. 2, a second major *in vitro* and *in vivo* autophosphorylation site of DNA-PKcs, S2056, was identified by immunoprecipitating DNA-PKcs from nuclear extracts prepared from irradiated HeLa cells and analyzing by mass spectrometry (Fig. 2). DNA-PKcs from irradiated HeLa cells was purified by immunoprecipitation and digested with Asp-N protease. The peptide mixture was treated or mock treated with alkaline phosphatase and analyzed by MALD-TOF mass spectrometry.

Shown in Fig. 2 is the mass spectra of phosphatase-treated (bottom trace) and the mock treated (top trace) peptide mixture. The loss of the peak with the m/z of 3511 with phosphatase treatment, and the presence of the peak corresponding to the unphosphorylated peptide (m/z of 3433) allowed the inventors to positively assign a phosphorylation site to a sequence of DNA-Pkcs between amino acids 2044-2072. This peptide was then sequenced by tandem mass spectrometry (as in Fig 1) to unequivocally identify S2056 as the site of phosphorylation.

[078] Mass spectrometry identified the following phosphopeptide sequence, SEQ ID NO: 5, DFSTGVQSYSYSSQDPRPATGRFRRREQR, which corresponds to amino acids 2044 to 2072 of DNA-PKcs (S2056 is underlined).

EXAMPLE 2

DNA-PKcs Fragments and GST Fusion Proteins Containing Autophosphorylated Sites

[079] 20 bp oligomer primers were designed and ordered from Operon (Alameda, CA) using SEQ ID NO: 4 (the nucleotide sequence of DNA-PKcs, GenBank Accession Number: P78527) to created primers to amplify cDNA sequence that encodes the phosphorylation sites, T2609 and S2056. Designed DNA-PKcs cDNA fragments that cover the phosphorylation sites in DNA-PKcs found by mass spectrometry were PCR amplified from the full-length DNA-PKcs cDNA (isolated and described by several of the inventors in Kurimasa et al., *Mol Cell Biol* **19**: 3877-3884, 1999) using the custom designed PCR primers under normal PCR thermal cycling conditions. The reactions were carried out using *pfu* DNA polymerase (Stratagene, La Jolla, CA) and GeneAmp 9600 thermocycler (Perkin Elmer). The amplified cDNA fragments were cloned in frame into GEX-KG vector (Guan & Dixon 1991 Analytical Biochem. 192:262-67) for fusion between domains of DNA-PKcs and GST.

[080] These clones were made to express the following residues of DNA-PKcs from the indicated corresponding cloned cDNA sequence: 1879-2182 cDNA (SEQ ID NO: 18), 1879-2267 cDNA (SEQ ID NO: 19), 2261-2700 cDNA (SEQ ID NO: 21), 2275-2702 cDNA (SEQ ID NO: 23), 2429-2702 cDNA (SEQ ID NO: 24), 2561-2700 cDNA (SEQ ID NO: 25), and 2600-2702 cDNA (SEQ ID NO: 26).

[081] Peptide fragments were expressed by the clones which encode the following peptides and correspond to the following residues of DNA-PKcs: 1879-2182 (SEQ ID NO:

6), 1879-2267 (SEQ ID NO: 7), 1879-2700 (SEQ ID NO: 8), 2261-2700 (SEQ ID NO: 9), 2500-2702 (SEQ ID NO: 10), 2275-2702 (SEQ ID NO: 11), 2429-2702 (SEQ ID NO: 12), 2561-2700 (SEQ ID NO: 13), and 2600-2702 (SEQ ID NO: 14).

[082] Several of these fragments were also made into glutathione-S-transferase (GST) fusion proteins. The PCR'd fragments were cut randomly and then fused with the GST protein using the commercially available GST fusion vector (Amersham Biosciences, Piscataway, NJ).

EXAMPLE 3

Preparation of Cellular Nuclear Extracts from Cells

[083] The preparation of nuclear extract from HeLa cells for the Examples that follow were made as generally described by Lees-Miller et al., *Mol Cell Biol* **10**: 6472-6481, 1990 and is herein described. The cells were washed twice with cold PBS, collected, and spun at 2000g for 5 min. The cell pellet is washed once with 5 ml LSB and spun again. The pellet is resuspended in 1ml LSB and transfer to a centrifuge tube. (LSB (low salt): 10 mM Hepes pH7.5, 25 mM KCl, 10 mM NaCl. 1mM $MgCl_2$, 0.1mM EDTA).

[084] After spinning down again, the volume of the cell pellet is estimated, then resuspended in 1x Pack cell volume (PCV) of LSB (with 50 mM NaF, 1mM DTT, 0.5 mM PMSF, and other protease inhibitors), set in ice 5 min, and freezed in liquid N_2. Thaw, and spin immediately at 10,000g for 10 min. Dispose of Supernatant (S10, cytosol fraction.

[085] The pellet is again resuspended in 1x pack nuclear volume (PNV) of LSB with 0.5M NaCl and 10 mM $MgCl_2$ (500 mM NaCl, 10 mM $MgCl_2$, 50 mM NaF , 1mM DTT, 0.5 mM PMSF), set in ice for 10 min. For this step, LSB (0.5 M salt): 10 mM Hepes pH7.5, 25 mM KCl, 500 mM NaCl. 10mM $MgCl_2$, 0.1mM EDTA. The pellet is spun down at 40,000g for 20 min, supernatant (P10 nuclear fraction). The collected P10 nuclear extract is mixed 1:1 with 2X Laemmli buffer (80mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1%BPB), then the sample is boiled at 100 °C for 3 min.

EXAMPLE 4

SDS Gels and Western Blots for DNA-PKcs

[086] Western blotting for the following Examples was performed as generally described by Chan et al., *Biochem Cell Biol* **74**: 67-73, 1996 and herein described.

[087] **Preparation of 8% low bisacrylamide gels.** Resolution gel mix (10 ml): 3ml 30% acrylamide, 0.4ml 2% bisacrylamide , 2ml 1M Tris-HCl pH8.8 , 3ml water , 100ul 10%

SDS, 100ul 10% APS, 8ul TEMED, allow to polymerize for at least 90 min. Stacking gel mix (5 ml): 3.4 ml water, 0.83 ml 30% acrylamide, 0.63 ml 1M Tris pH6.8, 50ul 10% SDS, 50ul 10% APS, 5ul TEMED, allow to polymerize for at least 30 min.

[088] **Gel Running.** Load the samples and run at 100V 1.5 to 2 hrs until BPB dye runs off. The electrophoresis running buffer is made as followed (per liter): 6 g Tris base, 28.8 g Glycine, 1 g SDS. Do not pH.

[089] **Transfer.** Remove gel from glass plates and place in 50ml electroblot, gently rock for 5-15 min. Electroblot (per liter): 5.8g Tris base, 2.93g glucine, 0.38 g SDS, 100ml methanol. For each gel to be blotted, prepare 2 squares of 3 mm filter paper cut to size of gel and place them in electroblot. Cut 1 square piece of nitrocellulose or PVDF membrane about the same size.

[090] Wet the PVDF membrane with Methanol, and equilibrate in electroblot for 5 min before use. For nitrocellulose, place directly in electroblot. Submerse blotting cassette and Scotchbrite pads in electroblot, assemble as follows: towards the black side of the cassette, scotchbrite (sponge), 3 mm filter paper, gel, nitrocellulose membrane, 3 mm filter paper, and then scotchbrite. Make sure that no air bubbles are trapped between the gel and the nitrocellulose membrane by smoothing out with gloved finger or rolling with a glass rod. Place assembly in transfer chamber with black side to black side; add the frozen cooling pack and fill to top with electroblot. Transfer at 100V (~250mA) for 1 hour on ice bath or 15V overnight at RT with gentle stirring of the electroblot.

[091] **Western Blot.** Place membrane in block solution (5% none fat milk powder in TTBS) for at least 60 min. TTBS: 10 mM Tris pH 8.0, 150 mM NaCl, 0.1% TWEEN-20.

[092] Incubate the blot with primary antibody for 1 to 2 hrs at RT, 1: 1000 dilution of purified DNA-PKcs antibodies in block solution. Wash blot with TTBS for 10 min, three times. Incubate blot with secondary antibody for 30 min at RT, 1: 5000 dilution of goat anti-rabbit HRP conjugate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TTBS.

[093] Wash blot with TTBS for 10 min, three times. Make up ECL solutions (Amersham Biosciences, Piscataway, NJ): 1ml of each A and B is sufficient for a blot. Immerse blot in ECL solution for 1 minute, remove from ECL solution, place in a sandwich of plastic wrap, expose blot to film in dark room for various times (5 sec to 10 min), and develop film.

EXAMPLE 5

Cellular Radiation Sensitivity and Defective DSB Repair in Cells Having T2609A and S2056A Mutant Proteins

[094] To investigate the biological significance of the T2609 and S2056 phosphorylation in relation to DNA repair, wild-type or mutant DNA-PKcs were tested for DNA DSB repair, and radiation survival. DNA-PKcs expression constructs were made and transfected into the DNA-PKcs-defective V3 CHO cell line (Kurimasa et al., *J Immunol.* 2000 Oct 1;165(7):3883-9). Stable V3 cell lines that were expressing wild-type DNA-PKcs (V3-F18), T2609A DNA-PKcs mutant proteins (V3-T2609A), S2056A DNA-PKcs mutant proteins (V3-S2056A) and S2056A/ T2609A DNA-PKcs double-mutant proteins (V3-S2056A/T2609A) were isolated and evaluated for DNA-PKcs protein expression levels, radiation sensitivity and DNA repair defects. The radiation sensitivity of these cell lines was examined by assaying for their colony forming ability after IR.

[095] The following was used to carry out site-directed mutagenesis and isolation of the mutant cell lines. The creation of the T2609A mutant (V3-T2609A) is herein described. The S2056A DNA-PKcs mutant (V3-S2056A) and S2056A/T2609A DNA-PKcs double-mutant (V3-S2056A/T2609A) were generated using the same methods but different primers.

[096] First, a 3kb Hind III fragment of DNA-PKcs cDNA covering T2609 was used as the template for generating the T2609A mutation of DNA-PKcs cDNA. Site-directed mutagenesis was performed using the Quik Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the forward (tccgatgtttgtggaggaccaggcctcccagggc) (SEQ ID NO: 27) and reverse (gccctgggaggcctggtcctccacaaacatcgga) (SEQ ID NO: 28) primers. The mutated DNA-PKcs cDNA fragment was assembled back into the full length DNA-PKcs cDNA as described in Kurimasa et al., *Mol Cell Biol* **19**: 3877-3884, 1999. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air by using alpha-MEM medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Transfection of the DNA-PKcs expression plasmid was performed with a calcium phosphate transfection system (Catalog no. 18306-019; Gibco-BRL, Gaithersburg, MD). For each 10^6 cells in a 100-mm tissue culture dish, 10 μg of the DNA-PKcs expression vector and 10 μg of the pSV2neo or pPur plasmid were transfected.

[097] T2609A DNA-PKcs expression plasmid together with pSV2neo plasmid were transfected into the V3 cell line. Forty-eight hours after transfection, cells were replated on selection medium containing 400 μg/mL of G418 for 10 days. After 7 to 21 days of selection, individual colonies were isolated and further cultured.

[098] Colony formation and FAR (Fraction of Activity Released) assays were performed as previously described (Kurimasa et al., *Mol Cell Biol* **19**: 3877-3884, 1999).

[099] Radiation survival assays generated survival curves for each cell line. These survival curves were obtained by measuring the colony-forming abilities of irradiated cell populations. Three hundred cells were plated on 60-mm plastic petri dishes and irradiated with 137Cs γ rays at 2 h after plating at a rate of 2.2 Gy/min to achieve a cumulative dose of 1, 2, 3, or 5 Gy. After 7 to 14 days, cells were fixed and stained with 1% crystal violet in a 70% ethanol solution, colonies containing more than 20 cells were scored, and the mean value for triplicate culture dishes was determined. Cell survival was normalized to plating efficiency of untreated controls for each cell type.

[0100] Referring now to Fig. 3A, nuclear extracts were prepared as described in Example 3 from V3 cells transfected with vector alone (V3-JM), full length wild-type DNA-PKcs (V3-WT) or DNA-PKcs containing the T2609A point mutant (V3-T2609A1) were analyzed for DNA-PKcs protein expression levels (top panel). Nuclear extracts (P10) were prepared as described in Example 3. 20-60 μg of each sample was analyzed by western blotting as described in Example 4. For analysis of the V3 cell lines, the DNA-cellulose pull-down method of Finnie et al. (*Proc Natl Acad Sci U S A* **92**: 320-324, 1995) was used to first concentrate DNA-PKcs onto the cellulose and then subjected to SDS-PAGE for western blotting. Hamster Werner proteins were analyzed to demonstrate equal sample loading (bottom panel).DNA-PKcs protein levels in V3 (lane 1) is undetectable due to low abundance of DNA-PKcs RNA, whereas, V3-WT (lane 3) and V3-T2609A (lane 4) showed similar levels of protein expression (Fig. 3A, top panel). On the bottom, the expression of *wrn* (another DNA repair protein) was determined to show the equal loading of the samples.

[0101] Complementation of human DNA-PKcs in V3 cells restored radioresistance resulting in survival that is comparable to wild-type CHO cells (Fig. 3B). In Fig. 3B, the V3 cells complemented with the S2056A mutation DNA-PKcs (V3-S2056A), T2609A mutation DNA-PKcs (V3-T2609A) and S2056A/T2609A double mutation DNA-PKcs (V3-S2056A/T2609A) showed a radiation sensitivity phenotype that was more severe than the wild type. Although expression of the S2056A and T2609A mutant proteins improved the survival of the V3 cell line, the survival rates were significantly lower than what was observed for V3-WT. The dose of IR required for 10% survival of the V3-JM, V3-S2056A, V3-T2609A, V3-S2056A/T2609A, and V3-WT cell lines was 1.2 Gy, 1.9 Gy, 2.4 Gy, 2.1 Gy, and 5 Gy, respectively (Fig. 3B). Thus, the D_{10} value is approximately four-fold higher

for V3-WT compared to the V3-JM non-complemented cells (that is 5 Gy/1.2 Gy), whereas resistance at the 10% survival level was increased by only two-fold in the V3-T2609A1 cells (5 Gy/2.4 Gy). However, the resistance at the 10% survival level is increased by about 2.5 times in the cell lines containing the S2056A mutation, thus showing the significance of phosphorylation of the S2056 site in radioresistance. At around 4 Gy, both the S2056Amutant and the double mutant drop below 1.0% survival rate, showing a greater radiosentivity is caused by mutating the S2056 site than the T2609 site alone. At a dosage of 5 Gy, the T2609A mutant survival rate was less than 1% while the wild type cells having functional DNA-PKcs showed a 10% or greater survival rate. Therefore, phosphorylation of T2609 and S2056 are shown to be important for cell viability in response to ionizing radiation (IR).

[0102] The presence of additional DNA-PKcs phosphorylation sites may explain why the V3-T2609A1 cells showed only a roughly two-fold increase in radiation sensitivity (at 10 Gy, Fig. 3B). In response to DNA damage, phosphorylation of multiple sites may be required for proper DNA-PK function, and thus explaining why mutation of T2609 produced only a two-fold increase in radiation sensitivity, but the mutation of S2056 produced a greater increase to radiation sensitivity.

[0103] In Fig. 3C, T2609A mutation compromises DSB repair as shown by the FAR (fraction of activity released) Assay which is a DSB rejoining assay. The FAR Assay is used to analyze the mobility of genomic DNA in the polyacrylamide gel electrophoresis (PAGE) and to measure the presence of DNA DSBs. The FAR assay uses pulsed field gel electrophoresis to indirectly measure the intactness of DNA in cells after gentle lysis in agarose plugs by quantifying the amount of DNA released from the wells immediately after IR exposure as a function of dose or after a period of incubation to allow repair after a given dose (Story et al., *Int J Radiat Biol* **65**: 523-528, 1994).

[0104] The V3-JM, V3-WT, V3-T2609A1 cell lines and the parental CHO cell line (AA8) were irradiated at the indicated dose and analyzed for the presence of DSBs by the FAR assay. DNA DSB repair activity following exposure to ionizing radiation was measured by two different methods: (i) rejoining kinetics, plotted as a function of time course after irradiation; and (ii) measure of residual DNA DSB lesions following exposure and recovery to three doses (0, 20, and 40 Gy) of 137 Cs γ rays. Exposures consisted of a dose rate of about 4 Gy/min on ice. Immediately following irradiation, the cold medium was replaced with medium that had been warmed to 37°C and the cells were placed in a 37°C tissue culture incubator for 4 h to allow for DNA DSB repair. The cells were then trypsinized on ice,

washed, suspended in agarose plugs, lysed, and electrophoresed. Residual DNA DSB lesions were determined by CHEF pulsed-field gel electrophoresis combined with a storage phosphorimaging system. Rejoined lesions were defined as the fraction of DNA that had regained sizes large enough to prevent migration during electrophoresis (DNA retained) and measured by comparing the intensity of fluorescence of the DNA fraction retained in the agarose well.

[0105] Referring now to Fig. 3C, there is a graph showing the results of the FAR assay utilized to evaluate the ability of each of the three cell lines to rejoin DSBs induced by IR. The graph plots the percentage of DNA retained in the well (which shows DSB rejoining capacity) versus total radiation dosage. The V3-WT and the parental AA8 CHO cell lines showed comparable DSB rejoining capacities (Fig. 3C, open (80% retained) and closed circles (85% retained), respectively) and . In contrast, the V3-JM and the V3-T2609A cells were significantly more defective in the rejoining of DNA DSBs at 4 hrs after irradiation (only 70% of DNA retained in well), consistent with previous observations (Kurimasa et al., *Mol Cell Biol* **19**: 3877-3884, 1999) and with the hypothesis that DNA-PKcs plays an important role in repair of DSBs. Together, these results show that phosphorylation of DNA-PKcs at T2609 is important for rejoining of DSBs and for cell survival in response to DNA damage caused by IR.

EXAMPLE 6

Generation and Specificity of Phosphospecific Polyclonal Antibodies to pT2609 Site and pS2056 Site

[0106] To study the *in vivo* phosphorylation status of DNA-PKcs at T2609 and S2056, phosphsphospecific antibodies were generated. The phosphospecific antibodies, pT2609Ab, recognizes phosphorylated T2609, and pS2056Ab recognizes phosphorylated S2056. pT2609 polyclonal antibodies were prepared by immunizing New Zealand white rabbits with a KLH-conjugated phosphopeptide, N'-TPMFVET[PO^3]QASQGT-C' (SEQ ID NO: 1). pS2056 polyclonal antibodies were prepared by immunizing New Zealand white rabbits with KLH-conjugated phosphopeptide, N'-QSYSYSS[PO^3]QDPRPAC-C' (SEQ ID NO: 2).

[0107] **KLH-Conjugated Phosphopeptide.** To create the KLH-conjugated phosphopeptide (SEQ ID NO: 1 and 2), the phosphopeptide was made by conventional oligonucleotide synthesis means by AgBio, Inc. (Fremont, CA). 10 mg of the phosphopeptide was dissolved in 0.05 mL DMSO first, then 1 mL PBS (PBS: 0.1 Phosphate

buffer + 0.15 M NaCl, pH 7.3) was added and mixed. Activated KLH (10 mg Soluble keyhole limpet hemacyanin) (Calbiochem #374817, EMD Biosciences, San Diego, CA) was mixed with the dissolved peptide and the mixture was incubated at 4 °C with gentle rotation for overnight or room temperature (RT) for 3 hours. At the end of the first incubation, 10 mg cysteine (L-cysteine HCl, Sigma, St. Louis. MO) in 2 mL PBS was added to the mixture, then vortexed briefly.

[0108] The phosphopeptides were conjugated to KLH by cross-linker Sulfo-SMCC (Pierce Biotechnology, Inc., Rockford, IL) which forms a disulfate bridge with the cysteine residues placed at the C' terminal of the synthesized peptides and cysteine residues on KLH. The mixture was then incubated with rotation at RT for 2 hours to block unreacted SMCC. Dialysis against 2L PBS was done with at least 2 buffer changes. Dialysis may proceed overnight. In place of dialysis, a SEPHADEX G-25 column (Amersham Biosciences, Piscataway, NJ) may be used again to desalting.

[0109] The peptide/KLH conjugate solution was collected in dialysis bag or in fractions (in case of G-25 gel) and 18 mg NaCl to each mL of the solution was dissolved to give the solution additional 0.3 M NaCl. The solution was centrifuged if particulates or precipitates observed. The protein concentration of the clear conjugate solution was measured by using A_{280nm} and a coefficient 1.4 = 1 mg/mL. The conjugate solution was then diluted to 1 mg/mL and aliquoted 1 mL of the peptide-KLH conjugate solution into tubes. Each tube contained 1 mg of the conjugate and was sufficient for one immunization dose for two rabbits. The tubes were stored at -20 °C until use.

[0110] The polyclonal antibodies were made by immunizing New Zealand white rabbits with the above KLH-conjugated phosphopeptide, (SEQ ID NO: 1 and 2) using standard methods well known in the art by AgBio, Inc (Fremont, CA). Crude rabbit serum was collected from the immunized rabbits. The KLH-conjugated phospho peptides were then mixed with adjuvant and were injected into a rabbit through intradermal injection to elicit immunogenic response. After repeated injection to boost the immunogenic response, samples of serum were collected and tested by ELISA assay (to determine the titer of the antibodies) until the titer reached to the peak. The antibodies were then harvested.

[0111] The phosphospecific antibodies were affinity purified through a phosphopeptide-conjugated Sepharose CL-4B column. SEQ ID NO: 1 was made as an unphosphorylated peptide, N'-PMFVETQASQGTC-C' which corresponds to the T2609 site

unphosphorylated. SEQ ID NO: 2 was made as an unphosphorylated peptide, N'-QSYSYSSQDPRPAC-C', to correspond to the S2056 site unphosphorylated.

[0112] The following protocol was used to affinity purify the pT2609 and pS2056 rabbit polyclonal antibodies. Two columns are needed. One column uses an unphosphorylated version of the phosphopeptides used to immunize the rabbits. A second column uses the phosphopeptides. Eluted IgGs are passed through the first unphosphorylated peptide column to deplete any IgGs that are not specific to pT2609 or pS2056 and then the flow-through is then passed through the second phosphopeptide column to affinity purify the polyclonal antibodies specific for pT2609 and pS2056.

[0113] To prepare the columns, dissolve the appropriate peptide (1 mg/per ml) in coupling buffer: 50 mM Tris pH 8.5, 5 mM EDTA. Pack 5 ml SulfoLink Coupling Gel (Pierce Biotechnology, Rockford, IL) in 10 ml disposable polystyrene column (Pierce Biotechnology), equilibrate the column with 6 column volumes of coupling buffer. Place the bottom cap to the column, and add 5 ml peptide solution (5 mg) to the column. Place top cap, and mix the column at RT for 15 minutes with gentle rotation. Set for 30 minutes without mixing. Drain buffer, wash column with 3 column volumes of coupling buffer. Place the bottom cap to the column, and add 5 ml 50 mM cysteine to the column. Place top cap, and mix the column at RT for 15 minutes with gentle rotation. Set for 30 minutes without mixing. Drain buffer, wash column with 16 column volumes of 1 M NaCl.

[0114] For affinity purification, wash both non-phospho and phosphospecific peptide columns with 5 column volumes of PBS. Load 15 to 30 ml crude rabbit serum onto non-phosphopeptide column in RT. Collect flow-through. This step is to remove none specific antibodies. Load the flow-through onto phosphospecific peptide column with PBS. Wash with 10 column volumes of PBS with 0.5 M NaCl. Elute with 3 column volumes of 0.1 M glycine pH2.5, collect 1 ml fraction and neutralize the pH with 50 μl 1M Tris pH 8.0, and check protein concentration by Bradford assay (Bio-Rad, Richmond, CA) and freeze in –20°C.

[0115] In Fig. 4A, GST fusion proteins, having fragments spanning amino acids 2500-2700 (SEQ ID NO: 10) fused to GST, were made according to Example 2. The fragments contained either the wild-type DNA-PKcs sequence or the T2609A point mutation. The GST fusion proteins were *in vitro* phosphorylated with purified DNA-PK as described in Example 2 and analyzed by Western blot according to Example 5. The wild-type DNA-PKcs sequence and the T2609A point mutation were probed with the pT2609pAb (top panel) and anti-GST (bottom panel) to show equal loading. The lack of any signal detected for T2609A

by the pT2609pAb of the invention in the Western blot shows that T2609 in DNA-PKcs is phosphorylated and that the pT2609pAb is specific for the phosphorylated T2609 site.

[0116] Referring now to Fig. 4B, the Western blot shows that pT2609pAb is specific to phosphorylated T2609 and does not recognize unphosphorylated DNA-PKcs. In Fig. 4B, affinity-purified pT2609 polyclonal antibody was used in immunoblotting with mock or autophosphorylated DNA-PKcs, lanes 1 and 2, respectively, and in the presence of excess unphosphorylated DNA-PKcs at the indicated molar excess ratios. Immunoblotting with unphosphorylated DNA-PKcs at 100 fold molar excess (relative to the phosphorylated DNA-PKcs) did not produce a detectable signal. Western blotting with pT2609pAb (top panel) and 25-4 DNA-PKcs monoclonal antibody (bottom panel) with mock or autophosphorylated DNA-PKcs (lanes 1 and 2, respectively) and with purified, unphosphorylated DNA-PKcs (lanes 3 to 9) at the indicated molar ratios relative to the amount of protein in lanes 1 and 2.

[0117] Referring now to Fig. 4C, a similar experiment shows that affinity purified pS2056Ab is specific to phosphorylated S2056. V3 (DNA-PKcs deficient CHO cells) complemented with either wild type human DNA-PKcs cDNA (V3-F18) or kinase dead mutant (V3-KA4) were subjected to mock or 10 Gy of ionizing radiation. DNA-PKcs protein was immunoprecipitated from nuclear extracts by 25-4 DNA-PKcs monoclonal antibody, and western blotted with the generated pS2056 rabbit polyclonal antibody (bottom panel). The blot was stripped and reprobed with the 25-4 DNA-PKcs monoclonal antibody (top panel). S2056 phosphorylation was diminished in V3-KA4 (kinase dead mutant) as compared to that of V3-F18 (wild type DNA-PKcs) indicating that DNA-PKcs autophosphorylation is responsible for IR-induced S2056 phosphorylation.

EXAMPLE 7

Localization of pT2609 Antibody to Site of DNA Double-Strand Breaks

[0118] In response to DNA damage, many DNA repair proteins form nuclear foci, presumably, the site of the DNA DSBs (Rogakou, *J Cell Biol* 1999 Sep 6; 146(5):905-16; Maser et al., *Mol Cell Biol.* 1997 Oct; 17(10):6087-96). To determine the status of DNA-PKcs in response to DNA damage, DNA-PKcs was examined by immunofluorescence microscopy. Immunostaining with a monoclonal antibody to DNA-PKcs produced strong signal throughout the nucleus, in both unirradiated and irradiated cells (not shown).

[0119] Fluorescent immunostaining showed that pT2609pAb produce foci only in irradiated but not unirradiated primary human skin fibroblasts (HSF). Immunofluorescence was performed as previously described in (Burma et al. 2001, *J Biol Chem* 276: 42462-

42467). In contrast, immunostaining with 25-4 (a commercial monoclonal antibody to DNA-PKcs from Neomarkers, Lab Vision Corp, Fremont, CA) produced strong signal throughout the nucleus, in both unirradiated and irradiated HSFs, because DNA-PKcs is a very abundant nuclear protein.

[0120] Because DNA-PKcs is a very abundant nuclear protein, it is not possible to distinguish any foci in response to DNA damage with the monoclonal antibody. However, with the pT2609 polyclonal antibody, foci can be clearly detected in response to IR treatment. The number and size of foci varied with the dose of IR and reaches a maximum with 10 Gy (data not shown). In addition, the kinetics of the pT2609pAb foci formation is similar to what was observed with the time-course western results in Fig. 5B.

[0121] To further confirm the localization of T2609 phosphorylation at DSB sites, we examined the colocalization of rabbit pT2609 polyclonal antibody foci with the p53 binding protein (53BP1) which has been previously shown to bind to the site of DNA DSBs (Rappold et al., *J Cell Biol.*, Apr 30;153(3):613-20 2001). Co-immunostaining with a 53BP1 monoclonal antibody (Rappold et al., *J Cell Biol.*, Apr 30;153(3):613-20 2001) and the pT2609Ab in unirradiated HSF cells did not produce any discernable foci above the background signal of the nucleus. 53BP1 monoclonal antibody was provide by Dr. Junjie Chen (Mayo Clinic, Rochester, MN). However, cells that were irradiated resulted in very discrete 53BP1 and pT2609pAb foci. Moreover, the 53BP1 and pT2609pAb foci co-localized with each other. Thus by virtue of co-localization with 53BP1 foci in response to DNA damage, it was demonstrated that the pT2609pAb binds and becomes activated at the site of DNA DSB *in vivo*.

EXAMPLE 8

T2609 is Phosphorylated in Response to Irradiation *in vivo*.

[0122] Referring now to Fig. 5A, 50 μg of HeLa nuclear extracts made from unirriadiated (lane 3) or cells irradiated with 25 Gy and harvested after a 30 min recovery period, were analyzed by western blotting with pT2609pAb (upper panel) or 25-4 monoclonal antibody (bottom panel). As shown in Fig. 5A, phosphorylation of T2609 is DNA damage inducible and was detected as early as 10 minutes and reached a maximum at approximately 30 minutes after treatment. T2609 is phosphorylated up to 4 hours after IR treatment detectable, after which the phosphorylation of T2609 is not detectable (Fig. 5A). Purified DNA-PKcs was mock (lane 1, control) or autophosphorylated (lane 2) and analyzed by western blotting using the pT2609 polyclonal antibody of the invention. HeLa cells were

either mock treated or irradiated with 10 Gy and allowed to recover for the indicated times. Nuclear extracts were western blotted with pT2609pAb (top panel) and then blots were stripped and reprobed with the 25-4 DNA-PKcs monoclonal antibody (Neomarkers, Lab Vision Corp, Fremont, CA) (bottom panel). Since phosphorylation of T2609 can be detected as early as 10 minutes after IR, this suggests that phosphorylation of DNA-PKcs is an early event in response to DNA damage and is consistent with the hypothesis that DNA-PK is required for the early and rapid phase of the "biphasic" model of DSB repair. This biphasic model was previously described in DiBiase et al., *Cancer Res* 2000 Mar 1;60(5):1245-53.

[0123] Phosphorylation of T2609 is also dose dependent, and can be induced with 2 Gy of IR and reaches a maximum or saturation with 10 Gy of IR. As shown in Fig.5B, HeLa cells were irradiated with the indicated dose of IR and allowed to recover for 30 min. Nuclear extracts were first analyzed by western blot with pT2609pAb (top panel) and then with 25-4 monoclonal to show equal loading (bottom panel). Since phosphorylation of T2609 can be observed with a dose as low as 2 Gy, these results suggest that phosphorylation of DNA-PKcs is very sensitive to the presence of DSBs in the genome. The inventors have also observed the same phosphorylation of T2609 in response to IR in a lymphoblastoid cell line (Jurkat), a glioma cell line (M059K) and in primary human fibroblasts (data not shown), and thus this event appears to be a general phenomenon that is not cell-type specific.

[0124] Phosphorylation at T2609 in response to DNA damage was further confirmed by immunoprecipitation with the pT2609pAb. As shown in Fig. 5C, pT2609 polyclonal antibody was used to immunoprecipitate DNA-PKcs from 500μg of unirradiated HeLa nuclear extract (lane 1) or extracts made from HeLa cells irradiated with 25 Gy and harvested after 30 min recovery period. DNA-PKcs was immunoprecipitated with the pT2609pAb only in the nuclear extracts prepared from irradiated cells but not from that of untreated cells, thus showing that the pT2609pAb is specific for phosphorylated DNA-PKcs.

[0125] In Fig. 5D, phosphorylation of T2609 in response to DNA damage can be inhibited with wortmannin treatment and is inducible in A-T cells indicating that DNA-PKcs autophosphorylation is responsible for T2609 phosphorylation *in vivo*. The activity of PI-3 kinase family members, including DNA damage responsible DNA-PK and ATM, are sensitive to low dose of wortmannin (Sarkaria et al., *Cancer Res.* 1998 Oct 1;58(19):4375-82). Since phosphorylation of T2609 is through an autophosphorylation mechanism, then one would expect phosphorylation to be sensitive to the effects of wortmannin. Treatment of

HeLa cells with 20 μm wortmannin, resulted in a decrease in the detectable levels of phosphorylated T2609 (Fig. 5D).

[0126] To determine whether the ATM (ataxia-telangiesctasia-mutated) kinase may phosphorylate T2609 in response to IR, ATM deficient lymphoblastoid cell line (L3) and ATM positive cell line (BT) were treated with ionizing radiation and probed with pT2609pAb. In response to IR, phosphorylation of T2609 was observed in both the ATM wild-type (BT) and mutant (L3) cell lines, thus phosphorylation of DNA-PKcs at T2609 is ATM-independent.

EXAMPLE 9

Phosphorylation of S2056 *in vivo*

[0127] The observed S2056 phosphorylation *in vivo* data is similar to that of T2609. The experiments described in the previous Example were performed using p2056 polyclonal antibody (pS2056pAb) to observe the phosphorylation of S2056 *in vivo*. Referring now to Fig. 6A, phosphorylation of S2056 induced by irradiation *in vivo* was observed in HeLa cells (lane 2), wild type fibroblasts (lane 4), as well as ATM (ataxia-telangiesctasia-mutated) deficient fibroblasts (lanes 6 and 7). S2056 phosphorylation in response to IR in ATM deficient fibroblasts also indicates that DNA-PKcs autophosphorylation is likely responsible for S2056 phosphorylation *in vivo*. The gel in Fig. 6A shows that the pS2056pAb detects phosphorylated DNA-PKcs only in irradiated cells (lanes 2, 4, 6 and 7) but not unirradiated cells (lanes 1, 3, 5 and 8). In addition, fluorescent immunostaining with pS2056 antibody show that pS2056pAb detects only phosphorylated DNA-PKcs and is localized at DSB sites (nuclear foci) only in irradiated but not unirradiated cells (data not shown).

[0128] Referring now to Fig. 6B, a main difference between T2609 and S2056 phosphorylation is that prolonged phosphorylation of S2056 can be detected upon DNA damage. S2056 phosphorylation can be detected six to eight hours after IR whereas T2609 phosphorylation is diminished after about 4 hours indicating the phosphorylation at T2609 and S2056 may have overlapping and distinct functions.

EXAMPLE 10

Generating pT2609 and pS2056 Monoclonal Antibodies

[0129] Specific mouse monoclonal antibodies against pT2609 and pS2056 were prepared using immunogens disclosed herein. Protocols for immunization and construction

of hybridomes may be found in U.S. Pat. No. 4,455,296 to Hansen et al and U.S. Pat. No. 4,364,933 to Kong et al and are hereby incorporated by reference. The screening process, is as described in Example 6. The cell lines and monoclonal antibodies recognizing only the phosphopeptides but not non-phosphopeptides are selected. Therefore, there is no need of affinity purification of Example 6 for the phospho specific mouse monoclonal antibodies. Monoclonal antibodies to phosphorylated T2609 (pT2609mAb) and phosphorylated S2056 (pS2056mAb) were generated according to the protocol herein described.

[0130] Female BALB/c mice were subcutaneously injected with 100 μg/mouse into one spot with the phosphopeptide once a month for 4 months. The phosphopeptide was added to incomplete Freund's adjuvant. Testbleeds from mice's tail were drawn once a month after sterilizing mice skin with 70% alcohol. After 4 months of immunization, mice were given a final booster injection 4 days before doing fusion. The phosphoprotein was injected without adjuvant. The polyclonal mouse serum was collected from the hearts. The mice were sacrificed and the spleens and NS-1 myeloma cells were harvested.

[0131] The fusion of the harvested mouse NS-1 cells and spleen cells (B lymphocyte cells) was carried out according to the following method. Prepare hypoxanthine-aminopterin-thymidine (HAT, Sigma) medium (300 ml 15% RPMI medium + HTA) two days before. Defrost NS-1 cells (ATCC, Manassus, VA) and cultured in 15% RPMI medium (Gibco BRL, Gaithersburg, MD) (with 15% Fetal Bovine Serum obtained from Hyclone (Logan, UT)). Prewarm 2 ml 50% PEG, 5 and 15 ml RPMI, and 15% RPMI-HAT medium at 37°C.

[0132] Rinse the spleen in 5 mL RPMI in 60 mm petri dishes five times. Collect the speen cells and put into 15 ml centrifuge tubes (4°C). Pass the spleen cells through 25-gauge needles three times and 21-gauge needles three times. Spin at 4°C, 1800 rpm, 5 minutes. Collect NS-1 cells (about 200 ml culture) and put into 50 ml centrifuge tubes.

[0133] Spin at 4°C, 1400 rpm, 5 minutes. Resuspend spleen cells with 5 ml RPMI medium and stand for 2 minutes. Transfer the supernatant of spleen cells into a new 50 ml tube and repeat spin at 4°C, 1400 rpm, 5 minutes and resuspend spleen cells with 5 ml RPMI medium and stand for 2 minutes. Re-suspend the NS-1 cells in 15 ml RPMI and transfer into one 50 ml tube. Spin NS-1 cells, resuspend pellets and spin, and then resuspend the pellets with 20 ml RPMI medium. Spin spleen cells, 4°C, 1800 rpm, 5 min, resuspend the pellets with 4 ml RPMI medium. Count cell numbers in 2 μL spleen cells in 88 μl ammonium chloride (incubate 5 min, lysis RBC) and 10 μl tryphan blue (before counting). Count number of cells in 5 μl NS-1 cells, 40 μl PBS and 5 μl tryphan blue.

[0134] Keep some spleen ($5x10^6$) and NS-1 ($1x10^6$) cells as control. Transfer optimum amount of NS-1 cells into spleen cell tube. The optimum amount is 1:5 = NS-1 cells:Spleen cells. Spin at 4°C, 1800 rpm, 5 min and completely remove the supernatant. Drop by drop over 1 min period, add 1 ml pre-warmed 50% PEG with continually shaking the tube, shaking 30 sec, stand for 1 min (37°C). Drop by drop over 5 min period, add 5 ml RPMI medium to the fusion mixture while gently agitating (37°C). Immediately add 15 ml RPMI medium over a 1 min period. Incubate the fusion mixture in 37°C water bath for 5 min. Spin down the cell pellets at 25°C, 1800 rpm, 5 min. Re-suspend the cell pellets with 15% RPMI-HAT medium ($1x10^6$ spleen cells/ml). Use normal mouse splenocytes as feeder cells (seeding before cell fusion). Seed the cell suspension in 96 well plate (200 μl/well), culture two weeks. Screen positive clones by dot blot assay.

[0135] The present examples, methods, procedures, treatments, specific compounds and sequences are meant to exemplify and illustrate the invention and should in no way be seen as limiting the scope of the invention. Any patents or publications mentioned in this specification are indicative of levels of those skilled in the art to which the patent pertains and are hereby incorporated by reference to the same extent as if each was specifically and individually incorporated by reference.